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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) **Method and Agent for the Turbidimetric or Nephelometric Determination of Analytes**

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ABSTRACT

The invention relates to methods and agents for the turbidimetric or nephelometric determination of analytes in liquids with the aid of an antibody-binding reaction. The method is characterized in that a polypeptide is reacted with antibodies or fragments thereof, the resulting coupling product is incubated with the analyte, and the resulting turbidity is measured.

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5 Method and agent for the turbidimetric or nephelometric
 determination of analytes

The invention relates to method and agent for the turbidimetric or nephelometric determination of analytes in liquids with the aid of an antibody-binding reaction.

Both immunoturbidimetry and immunonephelometry
5 are based on the interaction between antibodies and the antigen to be detected. This interaction results in the formation of high molecular weight aggregates which act as centres for scattering incident light. The scattering of the light is recorded as an increase in extinction in
10 immunoturbidimetry and as an increase in the intensity of scattered light at an angle $> 0^\circ - \leq 90^\circ$ relative to the incident light in immunonephelometry. Since the extent of the light scattering is approximately proportional to $1/\lambda^4$, the wavelength regions typically selected for
15 immunoturbidimetry and immunonephelometry are of short wavelength (320-360 nm). The theoretical bases of immunoprecipitation are described, for example, in Immunology 32, 445-457 (1977).

The optimal conditions for carrying out such
20 determinations, such as buffers, detergents, neutral salts, accelerators, etc., are described in the literature and vary slightly depending on the antibodies used in each case and on the antigens to be detected (Ann. Clin. Biochem. 20, 1-14 (1983)).

25 The great advantage of the immunoturbidimetry and immunonephelometry described to date is that the methods can be automated relatively simply. Immunoturbidimetry and immunonephelometry are described for many serum proteins (The Plasma Proteins, Vol. 2, Academic Press,
30 New York, pages 375-425 (1975), Automated Immunoanalysis, Part 1+2, R.F. Ritchie (ed.), Marcel Dekker Inc., New

York, Basel (1978)).

The detection limits reported in the state of the art differ and vary depending on the antibody and antigen. The detection limits for immunoturbidimetry are at about 5 mg/l, and for immunonephelometry are at about 1 mg/l. The upper limits for the measurement range for immunoturbidimetry and immunonephelometry are at about 200-500 mg/l, depending on the optimisation of the assay.

It is not possible to determine lower concentrations of analytes by immunoturbidimetry and immunonephelometry. Employed for this is, for example, latex agglutination which uses relatively large particles (diameter about 0.05-3 μ m) which are coated with antibodies or antigens depending on the principle of the assay. Detection limits < 1 μ g/l can be achieved with the latex agglutination technique. The typical upper limits for the measurement range are then, however, as much as 200-1000 μ g/l (0.2-1 mg/l).

The present invention is based on the object of providing methods and agents for the turbidimetric or nephelometric determination of analytes which are more sensitive than the known methods and which permit detection of antigens in the concentration range 0.1 - > 100 mg/l.

The invention relates to a method for the turbidimetric or nephelometric determination of analytes in liquids with the aid of an antibody-binding reaction, which is characterised in that a polypeptide is reacted with antibodies or fragments thereof, the coupling product resulting therefrom is incubated with the analyte, and the resulting turbidity is measured.

The invention further relates to an agent for the turbidimetric or nephelometric determination of analytes in liquids, which contains a coupling product composed of a polypeptide and antibodies or fragments thereof, preferably a coupling product composed of a globular protein and Fab' fragments.

It has been found, surprisingly, that polymerised antibodies which have been obtained, for example, by acid precipitation from antiserum provide a higher measurement signal than monomeric antibodies in the turbidimetric assay. However, there is variation in the degree of polymerisation of these antibodies and thus in the measurement signal during storage. To avoid this disadvantage, a defined, stable complex with multiple binding sites has been prepared by coupling natural IgG or antibody fragments, preferably Fab' fragments, to a molecular carrier.

Suitable molecular carriers are polypeptides such as proteins with a molecular weight of at least 10,000 D. Preferably used are globular proteins such as albumin, thyroglobulin, transferrin, haemocyanin or else polypeptides such as polylysine. It is likewise possible to employ the known avidin/biotin or streptavidin/biotin system, in which case biotinylated antibodies or fragments thereof form a stable complex with avidin or streptavidin.

The coupling to the antibodies or fragments thereof can be carried out in a variety of ways. Preferably employed for this purpose are bifunctional heterocrosslinkers, for example succinimidylmaleimide crosslinkers and related substances. The advantage of these crosslinkers is that unwanted coupling products are avoided, antibodies or antibody fragments are introduced stepwise, the antibodies or antibody fragments are coupled end-on, and the coupling products are easy to purify by chromatography.

The coupling products are generally prepared by the following steps:

1. cleavage of the antibodies, for example with pepsin,
2. purification of the F(ab')₂ fragments by chromatography,
3. reduction of the F(ab')₂ fragments,
4. isolation of the Fab' fragments by chromatography,
5. coupling of the crosslinker onto the carrier,

6. purification of the carrier-crosslinker conjugate by chromatography,
7. coupling of the Fab' fragments onto the carrier-crosslinker conjugate,
- 5 8. purification of the coupling product by chromatography.

The carrier-crosslinker conjugate can be prepared without problems in a batch process and be stored frozen in solution or lyophilised. It is likewise possible for
10 the F(ab')₂ fragments to be prepared in a batch process and to be stored as described above. Only steps 3, 4, 7 and 8 are then necessary for the actual preparation.

Compared with known reagents, in the turbidimetric and nephelometric assay the reagents prepared in
15 this way give distinctly higher measurement signals and thus allow the sensitivity of detection to be increased. The sensitivity of detection can be further optimised by the presence of known accelerators (for example polyethylene glycols). The reagents prepared in this way are
20 stable in solution, they can be employed without problems in automatic analysers, and they are thus also suitable for automated immunoturbidimetric determination of analytes.

Both the immunoturbidimetric and the immunonephelometric determination is carried out at 320-400 nm, preferably at about 340 nm. The determination can be carried out kinetically but preferably as endpoint reaction.

The method and agents according to the present
30 invention can be used to determine all antigens, that is to say substances which have a plurality of binding sites for antibodies. Examples of proteins of this type are transferrin, CRP, α_1 -microglobulin, RF, TBG, RBP, C3, C4, Igs etc. which occur in body fluids and are of diagnostic
35 relevance. These proteins are measured in the concentration range of 0.05 - > 100 mg/l, preferably 0.5 - 100 mg/l, without further prior dilution. Haptens which have only one binding site are amenable to competitive

determination using so-called polyhapten.

Example 1

Preparation of an anti-transferrin Fab'-albumin conjugate

3.2 g of sheep anti-transferrin IgG are taken up
5 in 150 ml of 0.9 % NaCl solution. The solution is
adjusted to pH = 4.5 with 1 M acetic acid. 80 mg of
pepsin are added to this solution, and the mixture is
incubated at 37°C for about 16 h. The reaction solution
is adjusted to pH = 7.0 with 1 M TRIS solution. The
10 reaction solution is purified by affinity chromatography
using a Sepharose[®] 4B column to which human transferrin is
covalently bonded. The bound F(ab')₂ fragments are eluted
with 0.1 M glycine/HCl (pH = 2.8). The protein-containing
fractions are pooled and adjusted to pH = 7.0 with 1 M
15 TRIS solution. The solution is concentrated to 30 ml in
an ultrafiltration cell. After photometric determination
376 mg of specific F(ab')₂ fragments are obtained.

16 ml of this solution are adjusted to pH = 4.9
with 1 M acetic acid, deaerated and blanketed with argon
20 and mixed with 0.9 ml of an 11% solution of cysteamine
hydrochloride in water. After incubation at 37°C for 2 h,
the Fab' fragments are purified by gel chromatography on
Sephadex[®] G-25 (150 mM NaCl, 20 mM phosphate buffer, pH =
7.0).

25 The protein-containing fractions are pooled.
After photometric determination, 190 mg of Fab' fragments
are obtained in 34 ml of solution. The solution is stored
in the cold under argon gas.

40.5 mg of bovine serum albumin are dissolved in
30 7 ml of water, and 1 ml of a solution of 30 mg of suc-
cinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
(SMCC) in dioxane is added. The reaction solution is
incubated at 30°C for 1 h and then purified by gel
chromatography on Sephadex[®] G-25. The protein-containing
35 fractions are pooled. After photometric determination,
38 mg of the coupling product are obtained in 14.8 ml of
solution. The Fab' solution is mixed under argon gas with

the solution of the coupling product and incubated at room temperature for 17 h.

5 The reaction mixture is purified by gel filtration chromatography (for example Superdex, Pharmacia/LKB, 150 mM KCl, 20 mM phosphate buffer, 1 mM EDTA, 0.02 % NaN₃, pH = 7.0). The fractions which contain the required product are combined.

Example 2

10 Turbidimetric immunoassay with anti-transferrin Fab'-albumin conjugate.

Reaction buffer:

0.1 M TRIS . HCl buffer, pH = 7.6, 6% polyethylene glycol 6000, 0.1% CHAPS, 0.1% TRITON[®] X-100, 0.02% NaN₃.

Fab'-albumin:

15 Prepared as in Example 1.

Transferrin:

Human transferrin was dissolved in 0.1 M TRIS . HCl buffer, pH = 7.6. Concentrations in the range to 100 mg/l transferrin were prepared by serial dilution.

20 Apparatus adjustment:

25 The assay is carried out as endpoint assay in an EPOS 5060 automatic analyser for clinical chemical assays. Firstly 40 μ l of sample (transferrin solution) and 200 μ l of reaction buffer are pipetted, mixed and preincubated at 37°C for 64 sec. Then 30 μ l of Fab'-albumin conjugate are pipetted in, mixed and the resulting extinction is measured after 288 sec. The apparatus cycle is set at 16 sec. The extinction at the end of the preincubation time, before addition of the start reagent,
30 is used to determine the sample blank. The resulting calibration plot (extinction/concentration) is depicted in Fig. 1.

Example 3**Preparation of an anti-CRP Fab'-albumin conjugate**

250 mg of lyophilised sheep anti-CRP IgG are dissolved in 20 ml of a 150 mM NaCl solution. The solution is mixed with 4 ml of 1 M sodium acetate buffer, pH = 4.9. The pH of the solution is adjusted to pH = 4.35 with 1 M acetic acid. 6.3 mg of pepsin are added, and the mixture is incubated at 37°C for ab : 16 h. The resulting F(ab')₂ fragments are purified by gel filtration chromatography (for example Superdex[®], Pharmacia/LKB, 20 mM potassium phosphate buffer, pH = 7.0, 150 mM KCl). The combined fractions contain 122 mg of F(ab')₂ fragments. The pool is concentrated to 12 ml in an ultrafiltration cell (12.3 mg/ml F(ab')₂).

6 ml of this solution (73.3 mg F(ab')₂) are mixed with 1 ml of 1 M sodium acetate buffer, pH = 4.9. The pH is adjusted to 5.0 with 1 M acetic acid. 0.35 ml of an aqueous 11% cysteamine hydrochloride solution is added to this solution. The reaction mixture is blanketed with argon gas and incubated at 30°C for 2 h. The resulting Fab' fragments are purified by gel filtration on Sephadex[®] G-25. 53 mg of Fab' fragments are obtained.

14.6 mg of bovine serum albumin are dissolved in 1.8 ml of water. 7.2 mg of SMCC in 0.18 ml of dioxane are added to this solution and incubated at 30°C for 1 h. The resulting BSA-SMCC conjugate is purified by gel chromatography on Sephadex[®] G-25. 14.3 mg of BSA-SMCC conjugate are obtained. The solution of the Fab' fragments is combined under argon gas with the BSA-SMCC conjugate solution and incubated at room temperature for 18 h. 0.25 ml of an 11% cysteamine hydrochloride solution is subsequently added. The reaction mixture is concentrated to 8 ml in an ultrafiltration cell. The reaction mixture is purified by gel filtration chromatography (for example Superdex[®], Pharmacia/LKB, 150 mM KCl, 20 mM phosphate buffer, 1 mM EDTA, 0.02% NaN₃, pH = 7.0). The fractions which contain the required product are combined (21 ml).

Example 4

Turbidimetric immunoassay with anti-CRP Fab'-albumin conjugate

Reaction buffer:

- 5 0.1 M TRIS · HCl buffer, pH = 7.6, 6% polyethylene glycol 6000, 0.1% CHAPS, 0.1% TRITON® X-100, 0.02% NaN₃,

Fab'-albumin:

Prepared as in Example 3

CRP:

- 10 Human CRP is dissolved in 0.1 M TRIS · HCl buffer, pH = 7.6. Concentrations in the range to 100 mg/l CRP are prepared by serial dilution.

Apparatus adjustment:

- 15 The assay is carried out as endpoint assay in an EPOS 5060 automatic analyser for clinical chemical assays. Firstly 20 µl of sample (CRP solution) and 220 µl of reaction buffer are pipetted, and the mixture is preincubated at 37°C for 64 sec. Then 30 µl of Fab'-albumin conjugate are pipetted in, mixed and the resulting extinction is measured after 288 sec. The apparatus cycle is set at 16 sec. The extinction at the end of the preincubation time, before addition of the start reagent, is used to determine the sample blank.

- 20 The resulting calibration plot (extinction/concentration) is depicted in Fig. 2.

Example 5

Preparation of an anti-transferrin Fab'-thyroglobulin conjugate

- 30 Anti-transferrin IgG (sheep) is treated with pepsin in analogy to Example 1. The F(ab')₂ fragments are purified by gel filtration chromatography. 1.3 ml of a solution of 41.5 mg of these F(ab')₂ fragments in phosphate buffer (20 mM phosphate, 150 mM KCl, pH = 7.0)

are mixed with 0.2 ml of 1 M acetate buffer, pH = 5.0, and blanketed with argon gas. 0.1 ml of an 11% cysteamine hydrochloride solution in degassed water is added and incubated at 37°C for 2 h. The resulting Fab' fragments
5 are purified by gel chromatography on Sephadex^G-25. The yield is 34.6 mg of Fab' fragments in 2 ml of elution buffer.

25 mg of thyroglobulin (pig) are dissolved in 3 ml of phosphate buffer (20 mM phosphate, 150 mM KCl, pH = 7.0). 0.1 ml of a solution of 27 mg/ml SMCC in dioxane is added, and the mixture is incubated at 30°C for 30 min. The reaction mixture is purified by gel chromatography on Sephadex^G-25. The solution of the Fab' fragments is added in portions to the coupling product
15 and incubated at room temperature overnight.

The resulting thyroglobulin-(Fab')_n conjugates are purified by gel filtration chromatography (150 mM KCl, 20 mM phosphate buffer, 1 mM EDTA, 0.02% NaN₃, pH = 7.0). The fractions which contain the required product
20 are combined (24 ml).

Example 6

Turbidimetric immunoassay with anti-transferrin Fab'-thyroglobulin conjugate

Reaction buffer:

25 0.1 M TRIS · HCl buffer, pH = 7.6, 5% polyethylene glycol 6000, 0.1% CHAPS, 0.1% TRITON[®], 0.02% NaN₃,

Fab'-thyroglobulin:

Prepared as in Example 5

Transferrin:

30 Human transferrin is dissolved in 0.1 M TRIS · HCl buffer, pH = 7.6. Concentrations in the range to 100 mg/l transferrin are prepared by serial dilution.

Apparatus adjustment:

The assay is carried out as endpoint assay in an EPOS 5060 analyser. Firstly 15 μ l of example (transferrin solution) and 235 μ l of reaction buffer are pipetted, and
5 the mixture is preincubated at 37°C for 64 sec. Subsequently 60 μ l of Fab'-thyroglobulin conjugate are pipetted in, mixed and the resulting extinction is measured after 288 sec. The apparatus cycle is set at 16 sec. The extinction at the end of the preincubation time,
10 before addition of the start reagent, is used to determine the sample blank.

The resulting calibration plot (extinction/concentration) is depicted in Fig. 3.

Example 7

15 Comparative experiment to determine transferrin

Reaction buffer:

0.1 M TRIS . HCl buffer, pH = 7.6, 5% polyethylene glycol 6000, 0.1% CHAPS, 0.1% TRITON® X-100, 0.02% sodium azide

Fab'-albumin:

20 Prepared as in Example 1

Anti-transferrin IgG:

Starting material of Example 1

Transferrin:

- Human transferrin is dissolved in 0.1 M TRIS .
25 HCl buffer, pH = 7.6. Concentrations in the range to 100 μ g/l transferrin are prepared by serial dilution.

Apparatus adjustment:

The assay is carried out as endpoint assay in an EPOS 5060 analyser. Firstly 20 μ l of sample (transferrin solution) and 235 μ l of reaction buffer are pipetted, and
30 the mixture is preincubated at 37°C for 64 sec. Subsequently 50 μ l of Fab'-albumin conjugate or anti-

transferrin IgG are pipetted in, mixed and the resulting extinction is measured after 288 sec. The apparatus cycle is set at 16 sec. The extinction at the end of the preincubation time, before addition of the start reagent, is used to determine the sample blank.

The resulting calibration plots (extinction/concentration) with Fab'-albumin conjugate and anti-transferrin IgG are depicted in Fig. 4. Fig. 5 shows a section of these calibration plots for low transferrin concentrations. The measurement signals obtained with the Fab'-albumin conjugate are distinctly higher than with natural anti-transferrin IgG under conditions which are otherwise the same.

Example 8

Nephelometric immunoassay with anti-CRP Fab'-albumin conjugate

Reaction buffer:

0.1 M TRIS · HCl buffer, pH = 7.6, 5% polyethylene glycol 6000, 0.1% CHAPS, 0.1% TRITON[®] X-100, 0.02% sodium azide.

Fab'-albumin:

Prepared as in Example 3

CRP:

Human CRP is dissolved in 0.1 M TRIS · HCl buffer, pH = 7.6. Concentrations in the range to 77.5 mg/l CRP are prepared by serial dilution.

Apparatus adjustments:

The assay is carried out as manual endpoint assay in a fluorescence spectrophotometer (HITACHI F 4000). The apparatus measures the intensities of scattered light at an angle of 90°. The intensities are reported as arbitrary units (a.u.). The wavelengths of excitation and emission are set at 340 nm. The slit width is set at 3 mm. After preceding zero adjustment, an expansion of

0-9999 a.u. is chosen.

200 μ l of sample and 2500 μ l of reaction buffer are pipetted into a 1 cm fluorescence cuvette, mixed and preincubated at room temperature for 90 sec. Subsequently
5 300 μ l of Fab'-albumin conjugate are pipetted in, mixed, and the increase in the scattered light intensity is followed for 10 min. The resulting measurements are plotted against the CRP concentrations.

The calibration plot (scattered light intensity
10 as a.u./concentration) is depicted in Fig. 6.

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Patent claims

- 5 1. Method for the turbidimetric or nephelometric determination of analytes in liquids with the aid of an antibody-binding reaction, which is characterised in that a polypeptide is reacted with antibodies or fragments thereof, the coupling product resulting therefrom is
10 incubated with the analyte, and the resulting turbidity is measured.
2. Method according to Claim 1, characterised in that a protein with a molecular weight of at least 10,000 D is used as polypeptide.
- 15 3. Method according to Claims 1 to 2, characterised in that a globular protein is used as polypeptide.
4. Method according to Claims 1 to 3, characterised in that albumin, thyroglobulin, transferrin, haemocyanin is used as polypeptide.
- 20 5. Method according to Claims 1 to 4, characterised in that Fab' fragments are used as fragments.
6. Agent for the turbidimetric or nephelometric determination of analytes in liquids, containing a coupling product composed of a polypeptide and antibodies
25 or fragments thereof.
7. Agent according to Claim 6, containing a coupling product composed of a globular protein and Fab' fragments.

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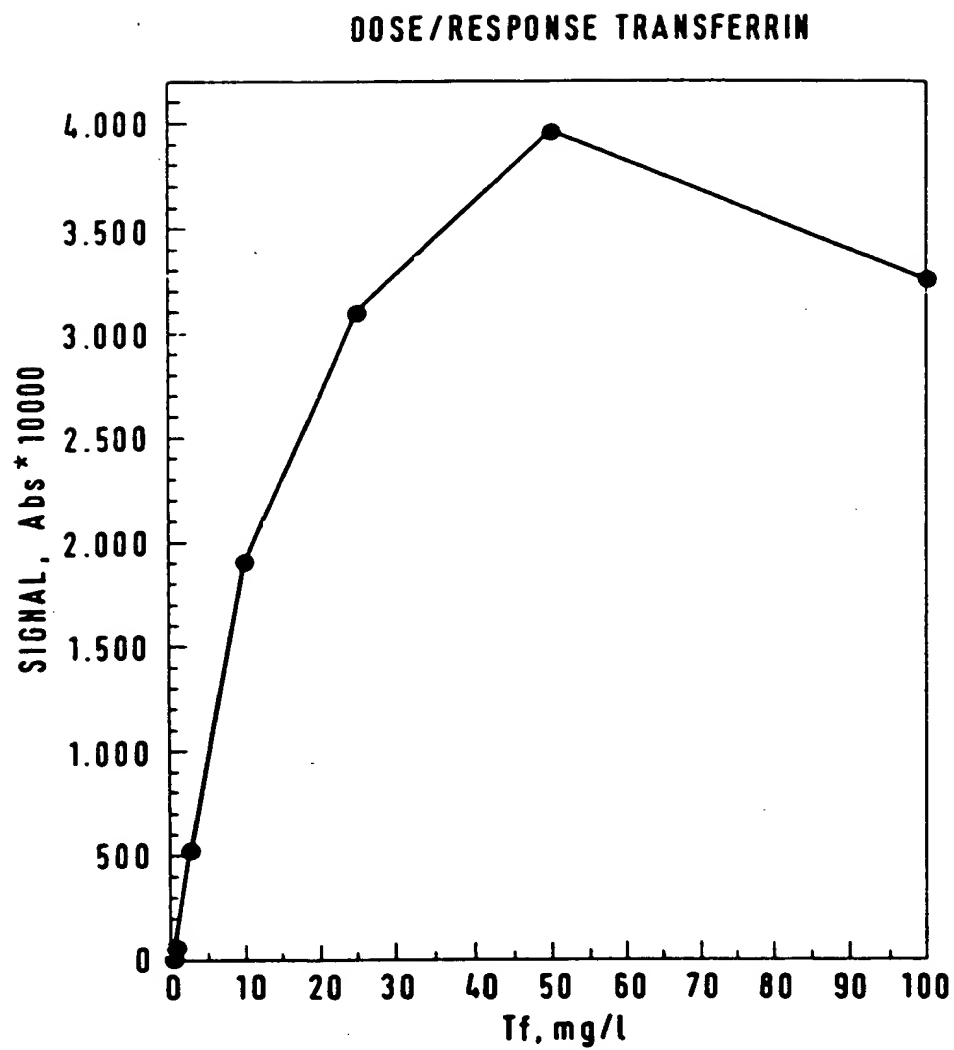


FIG. 1

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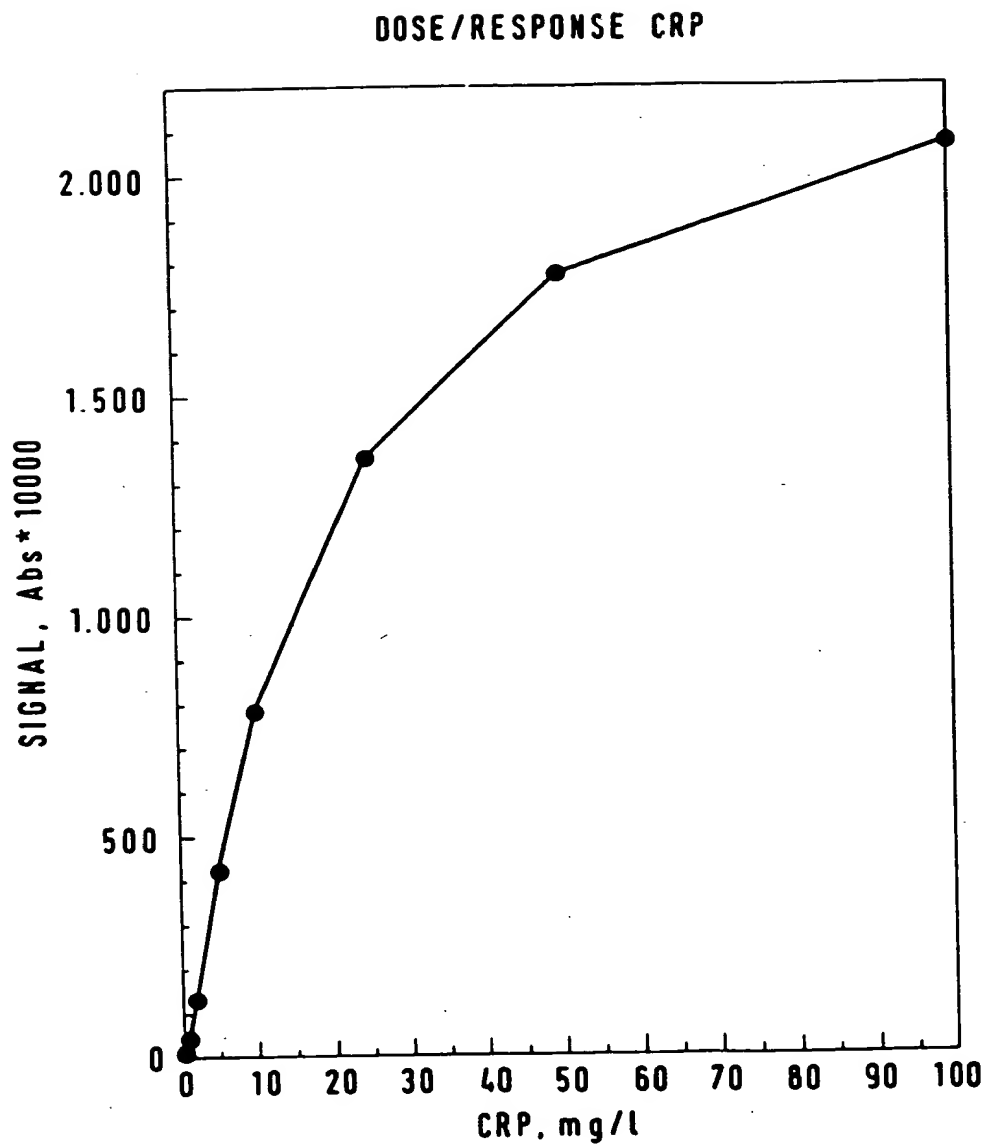


FIG. 2

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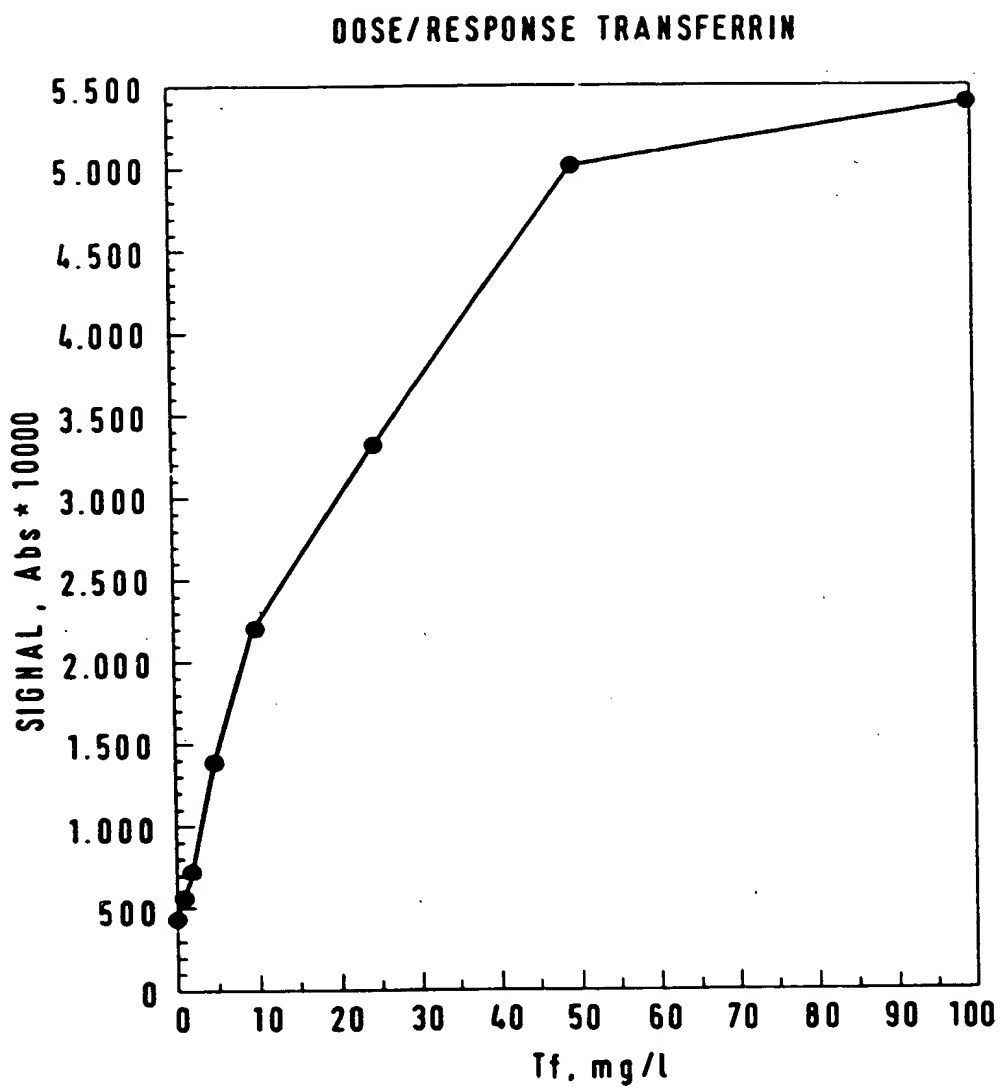


FIG. 3

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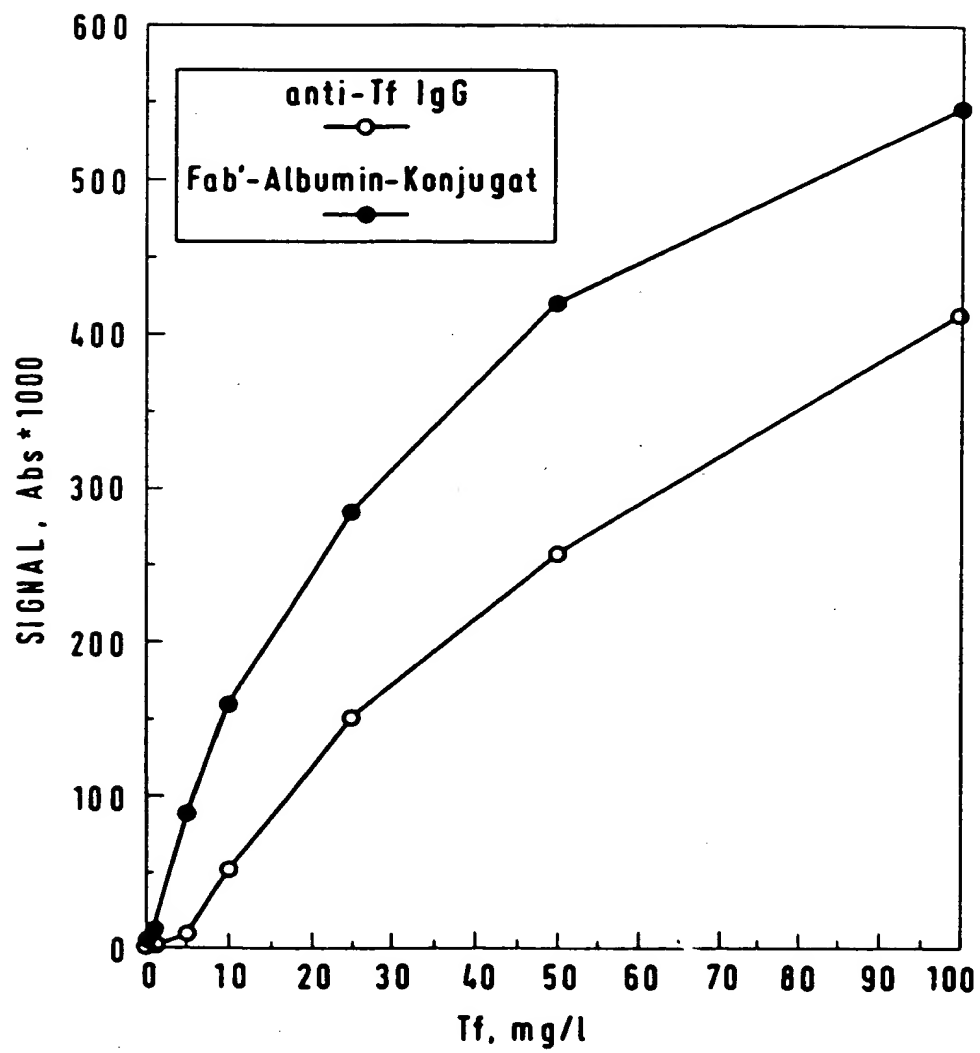


FIG. 4

Tf concentration

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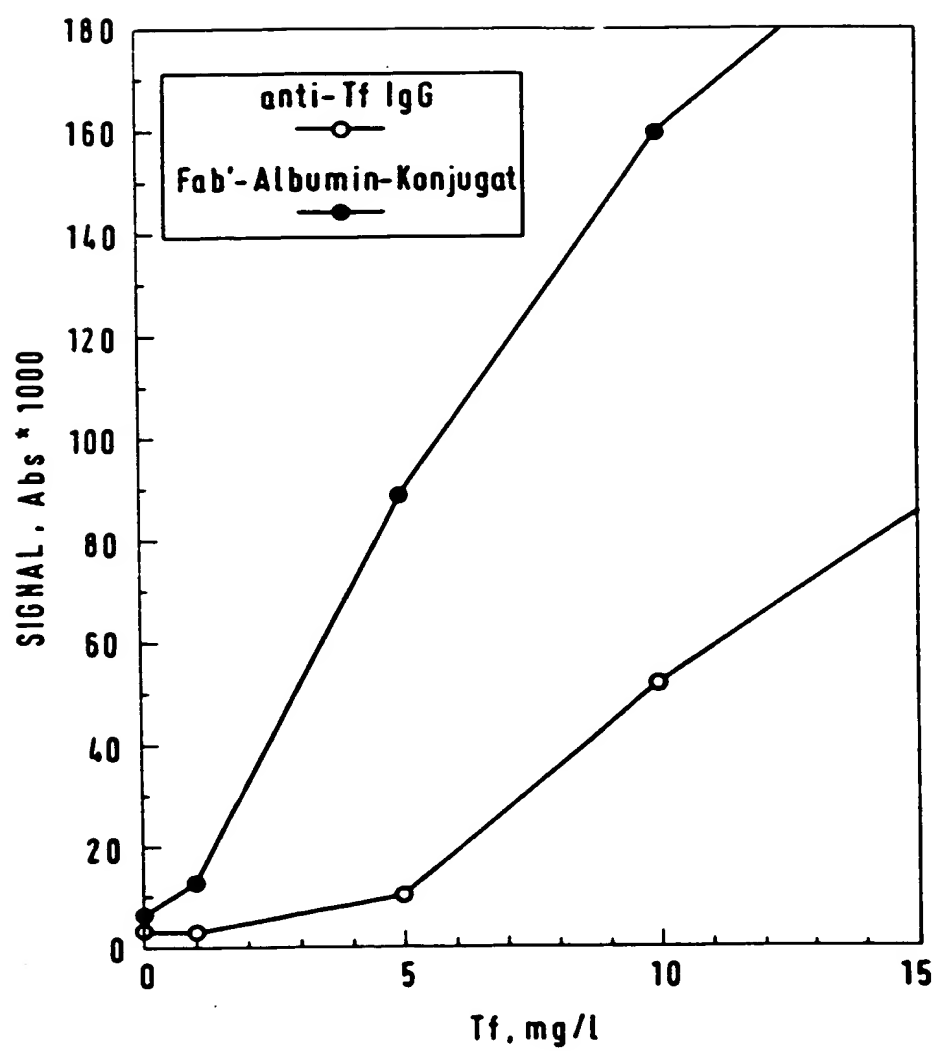


FIG. 5

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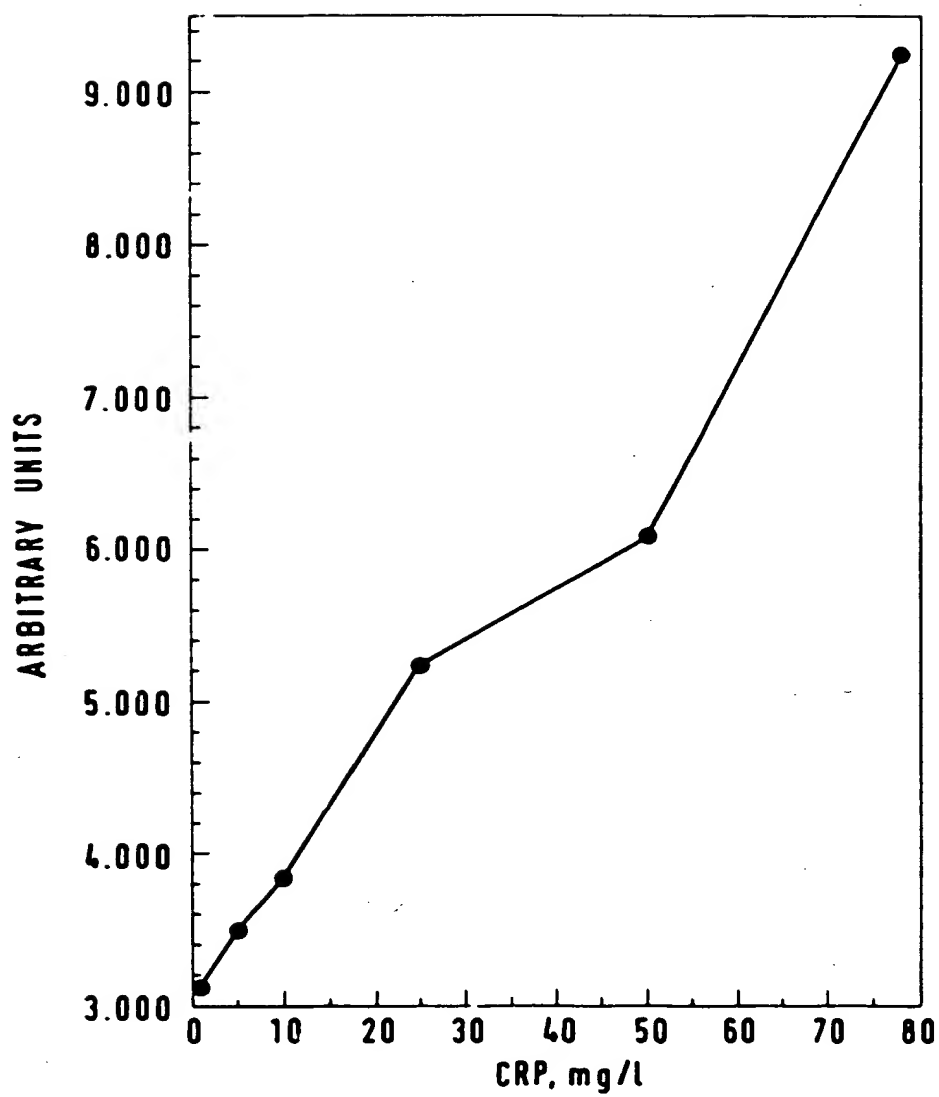


FIG. 6